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(71) Applicant *(for all designated States except US):* GIST-BROCADES N.V. [NL/NL]; Wateringseweg 1, P.O. Box 1, NL-2600 MA Delft (NL).

(72) Inventors; and
(75) Inventors/Applicants *(for US only) :* COX, Maria, Mathilde, Josephina [NL/NL]; Coendersstraat 34, NL-2613 SN Delft (NL). GERRITSE, Gijsbert [NL/NL]; De Hoef 38, NL-2995 VT Heerjansdam (NL). QUAX, Wilhelmus, Johannes [NL/NL]; Jan van Galenlaan 8, NL-2253 VB Voorschoten (NL).

(74) Agents: MATULEWICZ, Emil, Rudolf, Antonius et al.; Gist-Brocades N.V., Patents and Trademarks Department, Wateringseweg 1, P.O. Box 1, NL-2600 MA Delft (NL).

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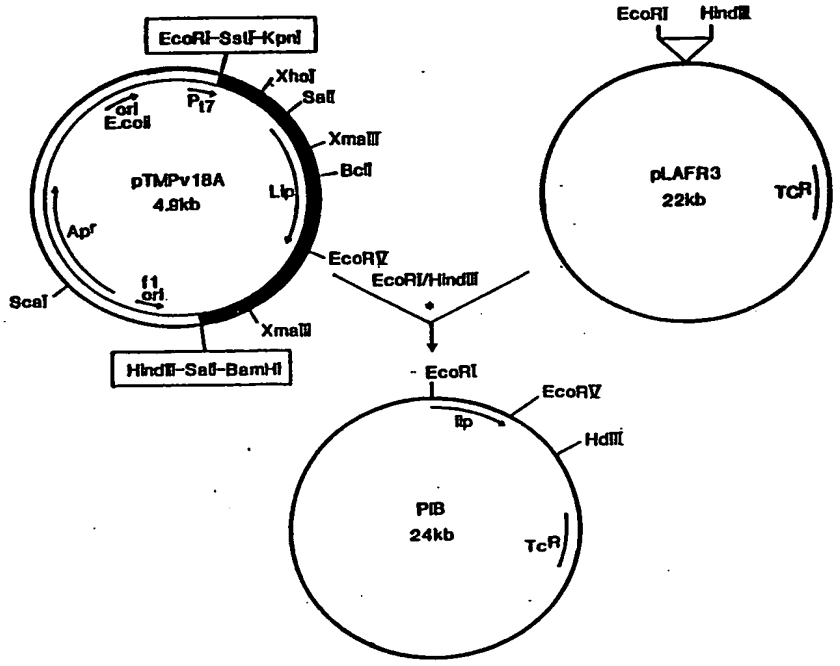

(57) Abstract

The present invention discloses the cloning and expression of a lipase modulator gene obtained from a class I *Pseudomonas* species. The expression product of the modulator gene is found to give rise to a considerable increase in lipase production especially upon homologous expression. The present invention provides a method for isolating a class I lipase modulator gene, an isolated modulator gene and a class I *Pseudomonas* transformed with such a gene. Finally the present invention discloses a derivative of plasmid pJRD215 which is segregationally stable in *Pseudomonas*.

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Cloning and expression of a lipase modulator gene from Pseudomonas pseudoalcaligenes

Technical field

The present invention describes the cloning and expression of a lipase gene in combination with a lipase modulator gene, both obtained from a class I Pseudomonas species, in an homologous class I Pseudomonas species.

Background of the invention

Lipases are enzymes capable of hydrolyzing lipids they are utilized in a wide range of applications such as fats and oil processing, detergents, diagnostic reagents etc.

Extracellular lipases (triacylglycerol acylhydrolases, E.C. 3.1.1.3) are produced by a wide variety of micro-organisms. Suitable microbial lipases have for example been disclosed in U.S. Patent No. 3,950,277, these lipases were obtained from such diverse microorganisms as Pseudomonas, Aspergillus, Pneumococcus, Staphylococcus, Mycobacterium tuberculosis, Mycotorula lipolytica and Sclerotinia.

It has turned out that especially Pseudomonas lipases have favourable characteristics for the desired applications. Pseudomonas species have therefore been extensively used for obtaining lipases. To increase lipase yield in fermentation several lipase genes have been cloned and expressed in both homologous and heterologous host strains. Examples of the Pseudomonas species from which lipase gene cloning has been reported are; Pseudomonas cepacia (EP 331376), Pseudomonas glumae (EP 464922), Pseudomonas pseudoalcaligenes (EP 334462), Pseudomonas fragi (EP 318775).

During this work it has been found that a lipase modulator gene was necessary to obtain lipase expression in an heterologous host.

EP 331376 describes the cloning and expression of a lipase gene obtained from Pseudomonas cepacia in P. cepacia.

It was found that no expression could be obtained when a second gene located downstream of the lipase gene was deleted. This gene was therefore reported to be essential for lipase production.

EP 464922 reports the cloning and expression of a lipase gene together with a gene encoding a protein reported to have a lipase-specific stabilizing/translocation function. The genes are obtained from Pseudomonas glumae and expression is preferably in heterologous systems. The stabilising protein is reported to differ greatly from the gene described in EP 331376 and therefor assumed to have a different function. WO 91/00908 reports the expression in a heterologous host of the lipase gene and the lipase modulator gene obtained from P. cepacia.

Lipase modulator genes, are reported to be essential for obtaining lipase production, however for an extensively investigated representative of class I Pseudomonas species: Pseudomonas fragi such a gene was not found. Another class I Pseudomonas lipase gene was described in EP 334462. EP 334462 reports the cloning and expression of the lipase gene from Pseudomonas pseudoalcaligenes in E. coli it can be concluded that for heterologous lipase production the lipase modulator gene was not essential.

The classification of Pseudomonas species is based on DNA-rRNA and DNA-DNA hybridization studies as reported by Palleroni et al. (Palleroni et al. Int.J.Syst.Bacteriol. 23 : 333 (1973)).
A more extensive overview can be found in Bergey's Manual of Systematic Bacteriology (Vol.1. Section 4 160-161 (1984). Eds N.R. Krieg and J.G.Holt. Williams and Wilkins, Baltimore/ London). This overview also reports that the classification is supported by morphological data and by 16S ribosomal RNA homology.

Generally a lipase modulator gene could not be detected in class I Pseudomonas species on the basis of homology with class 2 lipase modulator genes. Aoyama et al. FEBS Lett. 242

36-40 (1988) report the absence of such a gene in P. fragi. Recently a lipase modulator gene was reported (Ihara et al. J. Ferm. Bioengin. 73 (1992) 337-342) for a Pseudomonas species, which might belong to RNA homology group I, based on DNA homology (shown in table 3 and 4 of the present application). However, this gene was only used in E. coli and therein shown to be essential for the Pseudomonas lipase gene expression.

Summary of the invention

The present invention discloses a lipase modulator gene and the corresponding protein obtained from a class I Pseudomonas species.

The present invention also discloses Pseudomonas strains which have been transformed with a DNA sequence encoding a lipase and a sequence encoding a lipase modulator gene. These strains are preferably class I Pseudomonas strains and more preferably Pseudomonas pseudoalcaligenes strains.

The present invention further discloses a method for obtaining such transformed strains.

Furthermore the use of these strains for producing high amounts of lipase is disclosed.

The invention further discloses a vector derived from pJRD215 and which is segregationally stable in Pseudomonas. A method for obtaining such a vector is also disclosed.

Brief description of the drawings

Figure 1: Restriction map of plasmid **P1A**.

Symbols used are :

Km^r: gene encoding neomycin resistance of Tn5.

lip: gene encoding M1 lipase.

Furthermore a number of restriction sites are indicated.

Figure 2: Sequence of pJRD215 (derived from Davison et al. Gene 51 275-280 (1987)). The boxes indicate the direct repeat. At this site a recombination event occured, resulting in plasmid **P1Aδ** (shown in figure 3). The deletion was mapped by sequence analysis.

Figure 3: Restriction map of plasmid **P1Aδ**.

Symbols used are :

Km^r: gene encoding neomycin resistance of Tn5.

lip: gene encoding M1 lipase.

Due to the deletion plasmid P1Aδ is about 900 bp smaller than P1A, also several restriction sites are missing.

Figure 4: Construction and restriction map of plasmid **P1B**.

Plasmid pTMPv18A was described in EP 334462.

lip: gene encoding M1 lipase, location indicated by an arrow Tc^r: gene encoding tetracyclin resistance.

Plasmid pLAFR3 and derivatives harbour the replicon of RP4.

Figure 5: Restriction map of plasmid **P24A2δ**.

Symbols used are :

Km^r: gene encoding neomycin resistance of Tn5.

lip: gene encoding M1 lipase.

lim: gene encoding the M1 lipase modulator protein.

Figure 6: Restriction map of plasmid **P24B**.

lip: gene encoding M1 lipase, location indicated by an arrow

lim: gene encoding the M1 lipase modulator protein, location also indicated by an arrow.

Tc^r: gene encoding tetracyclin resistance.

Figure 7: Construction of plasmid **pBRint**.

amp^r: gene encoding ampicillin resistance.

tet^r: gene encoding tetracyclin resistance.

Figure 8: Physical map of the integration locus in the chromosome of Pseudomonas pseudoalcaligenes M1.

lip: gene encoding M1 lipase, location indicated by an arrow

lim: gene encoding the M1 lipase modulator protein, location also indicated by an arrow.
tet^r: gene encoding tetracyclin resistance.
lip$^-$: indicating an inactivated M1 lipase gene.

Figure 9: Construction of plasmid pUBint.
Plasmid pBHA-M1 was described in EP 334462 figure 15.
Km^r: gene encoding neomycin resistance of pUB110.
P_{HpaII}: HpaII promoter of plasmid pUB110.
[redacted] : insert derived from Pseudomonas pseudoalcaligenes M1, containing (part of) the lipase encoding sequence and part of the lipase modulator gene encoding sequence.

Figure 10: Physical map of the integration locus in the chromosome of Pseudomonas pseudoalcaligenes.
lip: gene encoding M1 lipase, location indicated by an arrow
lim: gene encoding the M1 lipase modulator protein, location also indicated by an arrow.
neo^r: gene encoding neomycin resistance of pUB110.
lim$^-$: indicating an inactivated M1 lipase modulator gene.

Detailed description of the invention

The recombinant DNA of the present invention is obtained by digestion of chromosomal DNA obtained from a strain of a Pseudomonas class I species. Representatives of class I Pseudomonas species are: Pseudomonas alcaligenes, Pseudomonas pseudoalcaligenes, Pseudomonas stutzeri, Pseudomonas aeruginosa, and Pseudomonas mendocina.

The chromosomal DNA is isolated using standard procedures as disclosed for example in Maniatis et al. Molecular cloning, Cold Spring Harbor Press, 1982 and 1989. A suitable digest is made and the fragments are cloned in a vector which is subsequently used to transform an E. coli. Selection is made on the basis of the presence of the lipase gene this can be performed using hybridization if suitable

probes are available. Alternatively it is possible to use an expression vector in which case it becomes possible to select for the presence of the desired genes using a suitable assay such as halo formation when lipase is screened for. Furthermore expression can also be monitored using immunological detection of the protein when suitable antibodies are available.

In the present invention a DNA library was obtained from Pseudomonas pseudoalcaligenes as a representative of class I Pseudomonads. The gene encoding the lipase was localized on a 2.0 kb PvuII fragment. The procedure has been described in EP 334462. Briefly, the lipolytic enzyme from the supernatant of Pseudomonas pseudoalcaligenes M-1 (CBS 473.85) was purified. After gel electrophoresis and blotting on Immobilon transfer membrane the N-terminal sequence was determined. A suitable probe was prepared based on this sequence. This probe was used in Southern hybridization experiments against chromosomal DNA which was isolated from Pseudomonas pseudoalcaligenes and which had been digested with several restriction enzymes. After size fractionation the fragments were cloned and again hybridized. A 2.0 kb PvuII fragment was found to contain the lipase gene. This fragment was sequenced and was also found to contain at least a part of a putative open reading frame.

This 2.0 kb fragment was cloned on an expression vector and the vector transformed to wild type Pseudomonas pseudoalcaligenes the resulting strain showed a 10-20 fold increase in lipase production.

A new expression vector was obtained containing the complete open reading frame which was localized downstream of the lipase gene on a 2.4 kb PvuII/BclI fragment the lipase gene and the open reading frame are found in their natural one operon sequence on this vector. Transformation of P. pseudoalcaligenes with this vector showed a 30 fold increase in lipase production over the expression vector containing only the lipase gene. This indicates that the open reading frame encodes a gene which modulates lipase expression.

The open reading frame was sequenced and found not to have a significant identity with the known class 2 lipase modulator genes, the identity was of the order of 30% at the amino acid level. The open reading frame also showed a 25 % identity with the lipase gene itself.

Upon inactivation of this modulator gene in the chromosome no lipase was produced. Cloning of the gene on an expression vector in such a host restored the lipase production.

This gene can therefore be considered to be the class I type lipase modulator gene. The cloning and expression of this modulator gene in wild type Pseudomonas pseudoalcaligenes did not have any effect on lipase production. However, when extra copies of the lipase gene were introduced into the cell, lipase production was significantly increased by addition of the modulator gene. From these experiments it is evident that the modulator gene is not required in a 1:1 ratio as compared with the lipase gene.

The present invention discloses for the first time that a class I lipase modulator gene increases the lipase productivity in a homologous host cell. Furthermore, it is also conclusively shown that the gene is not required in a 1:1 ratio compared with the lipase gene.

The present invention discloses a method for obtaining lipases comprising:

- cloning of a lipase gene and a lipase modulator gene obtained from a class I Pseudomonas species in a strain of an homologous Pseudomonas species,
- culturing of the recombinant strain under conditions wherein the lipase is expressed,
- isolating the lipase from the culture.

The host cells of the present invention and the source of the lipase modulator gene are preferably selected from the class I Pseudomonas species, Pseudomonas alcaligenes, Pseudomonas pseudoalcaligenes, Pseudomonas stutzeri,

Pseudomonas aeruginosa, and Pseudomonas mendocina. Mutants of these strains can also be employed.

In a most preferred embodiment Pseudomonas pseudoalcaligenes is the host strain.

The lipase gene and the lipase modulator gene can be cloned on a replicable expression vector they can however also be inserted in the chromosome of the host strain. Optionally the chromosomal lipase gene and the lipase modulator gene can be inactivated before the cloned genes are introduced. This is especially important when the production of mutated lipase is performed. The presence of the wildtype gene would then give rise to a significant contamination of the product. The copy number of the gene can be regulated by the choice of the vector.

The lipase and the lipase modulator genes are found to act both in cis and in trans. They are also found to be in one operon. It is therefore preferable to clone the genes in one operon. It is also possible to clone the genes on two separate vectors thereby enabling independent regulation.

The present invention further discloses a method for obtaining a plasmid which is segregationally stable in Pseudomonas.

This method comprises:

- repeated dilution of the transformed Pseudomonas strain in medium without antibiotics,
- followed by incubation periods in the presence of antibiotics.

The invention discloses a segregationally stable derivative of pJRD215 herein called p1Aδ. Sequence analysis shows that a fragment of 900 basepairs between two direct repeats has been deleted.

The following examples are meant to illustrate but not to limit the present invention.

Experimental

Colonies were grown on agar plates by using tributyrin (Merck) or castor oil as a substrate (described by Laurence et al. Nature 191 (1967) 1264-1265).
Plates were incubated at 30°C during 48-72 hours.

The clear zones that appear indicate lipolytic activity. A linear relationship can be observed when the logarithm of the enzym concentration is plotted against the diameter of zone of intensification.

Colonies were also grown on agar slices containing a defined volume of agar medium. After growth, the full grown colony is placed on a plate containing tributyrin. After an incubation period of 24 hours a zone of intensification can be observed. The diameter of this zone shows a linear relation with the logarithm of the level of enzym produced by the colony. The latter was observed when determining the activity of the supernatant of the strains grown in a lab fermentor as described in EP 334462, example 10.

Strains and plasmids described in this patent application are listed in table A.

Table A

Strains	Genotype	Reference
P.pseudoalcaligenes		CBS473.85
P.pseudoalcaligenes	R^-M^+	this patent
P.pseudoalcaligenes	$R^-M^+lip^-$	this patent
P.pseudoalcaligenes	$R^-M^+lim^-$	this patent
Plasmids	**Genotype**	**Reference**
pTZ18R	Ap^r, 2.9 kb, E.coli ori	Mead et al.
pTMPv18A	Ap^r, lip gene, 4.9 kb, E. coli ori	EP 0334462
pTZ18B24	Ap^r, lip gene, lim gene, 5.3 kb, E.coli ori	this patent
pJRD215	Km^r, RSF1010 ori, BHR, 10.2 kb, IncQ	Davison et al.
P1A (Figure 1)	Km^r, RSF1010 ori, BHR, lip gene, 12.2 kb, IncQ	EP 0334462
P1Aδ (Figure 3)	Km^r, RSF1010 ori, BHR, lip gene, 0.9 kb deletion, 11.3 kb, IncQ	this patent
pLAFR3	Tc^r, RP4 ori, BHR, 22 kb, IncP	Stascawicz et al.
P1B (Figure 4)	Tc^r, RP4 ori, BHR, 24 kb, lip gene, IncP	EP 0334462
P24A2δ (Figure 5)	Km^r, RSF1010 ori, BHR, lip gene, lim gene, 0.9 kb deletion, 11.7 kb, Inc Q	this patent
P24B2 (Figure 7)	Tc^r, RP4 ori, BHR, 24.4 kb, lip gene, lim gene, IncP	this patent
pBR322	Ap^r, Tc^r, 4.4 kb E.coli ori	Bolivar et al.
pBRint (Figure 8)	Tc^r, _ lip gene, 5.0 kb, E.coli ori	this patent

Table A (continued)

pUB110	Km^r, 4.5 kb, Bacillus ori	Gryczan et al.
pUBint (Figure 10)	Km^r, 4.0 kb, _ lim gene Bacillus ori	this patent
pBHA-M1 (Figure 10)	Km^r, Ap^r, 9.5 kb, Bac ori E.coli ori	EP 0334462
pBHM1 (Figure 10)	Km^r, 5.5 kb, Bac ori lip gene	this patent

Examples

Example 1

Cloning of the lipase gene and the lipase modulator gene from Pseudomonas pseudoalcaligenes M1 in Pseudomonas pseudoalcaligenes M1

The lipase gene from Pseudomonas pseudoalcaligenes M1 was cloned in E. coli as described in patent application EP 334462. In order to achieve homologous gene expression the SstI/HindIII lipase gene containing fragment of pTMPv18A (EP 334462: figure 11) was cloned into pJRD215 (Davison et al. Gene 51 (1987) 275-280) SstI and HindIII restriction sites. A restriction map of this plasmid, called P1A, is shown in Figure 1.

A stable derivative of this plasmid P1A was isolated after repeated dilution in medium without antibiotics followed by incubation periods. Characterization of the

obtained derivative revealed that a deletion of about 900 basepairs had occured, as shown in Figure 2. Surprisingly this plasmid, named P1Aδ, was much more stable than plasmid P1A. A restriction map of plasmid P1Aδ is shown in Figure 3. The improved segregational stability is shown in Table 1:

Table 1

Strain Pseudomonas pseudo alcaligenes M1 conaining plasmid	Segregational stability			
	Day 1	Day 2	Day 3	Day 4
P1A	83%	71%	5%	4%
P1Aδ	100%	100%	100%	100%

Cells were grown in a 2xTY culture without antibiotics, at O.D.=1, about 10^8 cells were inoculated into 2xTY medium and grown for 24 hours (=day 1), the culture was then diluted a thousand times and incubation was prolonged for 24 hours (=day 2) and so on. Colonies were first grown on plates without antibiotics for 24 hours at 30°C. Subsequently the colonies were replica-plated to plates containing 10 mg/l neomycin.

The EcoRI/HindIII lipase gene containing fragment of pTMPv18A was cloned into pLAFR3 (Staskawicz et al., J. Bacteriol. 169 (1987) 5789-5794) EcoRI and HinDIII restriction sites. A restriction map of this plasmid, called P1B, is shown in Figure 4.

As described in EP 334462, we also cloned BclI fragments of Pseudomonas pseudoalcaligenes M1 in E. coli. One of these clones, a 1.7 kb BclI fragment, appeared to contain the 3' region of the lipase gene and 1.2 kb of the downstream sequence. In order to investigate whether class I Pseudomonas

species also contain lipase modulator genes, both fragments (the 1.7 kb BclI and the SstI/HindIII (2.0 kb) lipase gene containing fragment of pTMPv18A) were combined in one expression cassette, resulting in clone pTZ18B24 (figure not shown). An internal PvuI/EcoRI fragment was exchanged with Pseudomonas expression cassette P1Aδ resulting in expression cassette P24A2δ. A restriction map of this plasmid is shown in Figure 5. The DNA sequence of the entire insert is shown in the Sequence Listing and contains two open reading frames one encoding the lipase gene and the other encoding a putative lipase modulator gene hereafter described as lipase modulator gene.

The EcoRI/HindIII lipase and modulator gene containing fragment of pTZ18B2.4 was also cloned into pLAFR3 EcoRI and HinDIII restriction sites, resulting in plasmid P24B2. The restriction map of this plasmid is shown in Figure 6.

All expression cassettes were introduced into Pseudomonas pseudoalcaligenes M1 using electroporation (Wirth et al. Mol. Gen. Gen. 216 (1989) 175-177).

Example 2

Chromosomal inactivation of both lipase and modulator gene of Pseudomonas pseudoalcaligenes

Suicidal integration plasmids, which are unable to replicate in Pseudomonas pseudoalcaligenes but able to replicate in other microorganism, were used to inactivate the lipase gene and the lipase modulator gene in the chromosome of Pseudomonas pseudoalcaligenes M1.

Inactivation of the lipase gene

An internal PvuI-PstI fragment of the lipase gene was cloned on suicide plasmid pBR322 (Bolivar et al. Gene 2 (1977) 95-113), able to replicate in E. coli, wherein the 38 N-terminal and the 49 C-terminal aminoacids of the lipase

coding sequence are missing (hereafter described as pBRint).

Detailed information about the construction of pBRint, derived from pBR322 and pTMPv18A (described EP 334462), is shown in Figure 7. Pseudomonas pseudoalcaligenes M1 R^-M^+ (restriction negative, modification positive) was transformed with pBRint. Several tetracycline resistent (5 mg/l) colonies were selected. They were all lipase negative, demonstrated in lacking a clearing-zone on castor oil (0.5%) agar plates and in a diminished clearing-zone on tributyrin (2%) agar plates. Pseudomonas pseudoalcaligenes M1 R^-M^+ itself gives rise to a clear halo on both types of agar plates. Southern analysis of two independent lipase negative electroporants revealed that the integration was established through a single cross-over event. As a result of this event the situation in the chromosome of Pseudomonas pseudoalcaligenes undergoes changes, outlined in Figure 8.

Inactivation of the lipase modulator gene

The internal EcoRV-PvuII fragment of the lipase modulator gene was cloned on suicide plasmid pUB110 (Gryczan et al. J. Bacteriol. 134 (1978) 318-329), able to replicate in almost all Bacillus species, wherein the 94 N-terminal and 107 C-terminal amino acids of the lipase modulator coding sequence are missing (hereafter described as pUBint).

Detailed information about the construction of pUBint, derived from pBHA-M1 (described in EP 334462), is shown in Figure 9.

Pseudomonas pseudoalcaligenes M1 R^-M^+ was transformed with pUBint. Several neomycin resistent (10 mg/l) colonies were selected. They were lipase negative, demonstrated in lacking a clearing-zone on castor oil (0.5%) agar plates and in a diminished clearing-zone on tributyrin (2%) agar plates. Southern analysis of three independent lipase negative electroporants revealed that the integration was established through a single cross-over event. As a result of this event

the situation in the chromosome of Pseudomonas pseudoalcaligenes undergoes changes, outlined in Figure 10.

Complementation

Complementation studies were performed with low copy number plasmids P1B, containing only the complete lipase gene and P24B2, containing both the complete lipase gene and modulator gene, and high copy number plasmids, P1Aδ containing only the complete lipase gene and P24A2δ containing both the complete lipase and modulator gene. The results of the complementation study are shown in Table 2. From these experiments it can be concluded that both the lipase and the lipase modulator gene, need to be intact for the total complementation of the lipase activity of both the lipase gene and lipase modulator gene inactivated strains.

Example 3

Lipase expression of transformed Pseudomonas pseudoalcaligenes M1

The lipase gene was expressed in heterologous host organisms (as described in EP 334462). However lipase expression levels were extremely low compared with the levels obtained in Pseudomonas pseudoalcaligenes M1. Therefor homologous gene expression was further developed.

The transformants were tested for their lipase production both on agar plates containing tributyrin and/or castor oil and after fermentation in olive oil based media as described by Odera et al. J. Ferment. Technol. 64 (1986) 363-371.
Results are shown in Table 2.

The improvement achieved by the introduction of multiple copies of the lipase gene expression cassette is 20

fold compared with the level of lipase produced by the parent strain, Pseudomonas pseudoalcaligenes M1.

However the expression level can be further improved by cloning the lipase modulator gene in the expression cassette as well.

From these data it can be concluded that the lipase modulator gene is not necessary in a 1:1 ratio but does become a limiting factor when lipase expression is increased over 20-fold.

Although the lipase modulator gene is necessary for lipase production the chromosomal copy of the gene is sufficient to allow an increase of 2000%. Only at this point introduction of additional modulation gene copies will result in higher lipase production.

It is unknown yet what the exact function of this gene might be, but our data suggest a chaperone like function, whereas only very low levels of the gene product seem to be sufficient for the secretion of large amounts of lipase.

Table 2

Lipase Productivity of Transformed Pseudomonas pseudo alcaligenes M1 Strains Containing the M1 Lipase Gene or the M1 Lipase Gene and the Lipase Modulator Gene		
Broad host range vector	Strain Genotype	Lipase productivity in %
-	M1	100
P1Aδ	M1	2000
P1B	M1	400
P24A2δ	M1	3000
P24B2	M1	400
-	M1:R$^-$M$^+$ *	≈50
-	M1:R$^-$M$^+$lip$^-$ *	≈0
P1Aδ	M1:R$^-$M$^+$lip$^-$ *	≈20
P1B	M1:R$^-$M$^+$lip$^-$ *	≈10
P24A2δ	M1:R$^-$M$^+$lip$^-$ *	≈1500
P24B2	M1:R$^-$M$^+$lip$^-$ *	≈200
-	M1:R$^-$M$^+$lim$^-$ *	≈0
P1Aδ	M1:R$^-$M$^+$lim$^-$ *	≈20
P1B	M1:R$^-$M$^+$lim$^-$ *	≈10
P24A2δ	M1:R$^-$M$^+$lim$^-$ *	≈1500
P24B2	M1:R$^-$M$^+$lim$^-$ *	≈200

* The growth rate of these strains is decreased probably due to a pleiotropic mutation

Example 4

Homology comparison of the lipase gene and lipase modulator gene with other lipase and lipase modulator genes

Lipase and lipase modulator gene sequences were compared using computer analysis. In order to determine homology with uncharacterized lipase genes, we used a molecular enzyme screening assay, as described in EP 334462.

From this work it was concluded that Pseudomonas species belonging to the same RNA homology group as P. pseudoalcaligenes show a rather strong homology, whereas Pseudomonas species belonging to a different RNA homology group (Palleroni, 1973) and other bacterial species show no hybridization at all. A correlation between both methods was well established, which makes it possible to determine homology with uncharacterized lipase genes.

Sequence comparison of the lipase gene

The sequences of several Pseudomonas lipase genes have been published. Computer analysis of these sequences compared with the Pseudomonas pseudoalcaligenes M1 reveals an identity of 81% for P. aeruginosa (EP 334462), also 81% for a Pseudomonas species (Ihara et al. J. Biol. Chem. 266 (1991) 18135-18140), 56% for P. fragi (Aoyama et al., FEBS Lett. 242 (1988) 36-40, Kugimiya et al., Biochem. Biophys. Res. Commun. 141 (1986) 185-190), all three probably belonging to RNA homology group I, 52% for P. cepacia (Jorgensen et al., J. Bacteriol. 173 (1991) 559-567) and 59% for P. glumae (PCT 91/00910), both belonging to RNA homology group II (Table III) and no homology at all with the lipase gene from S. hyicus (Götz et al., NAR 13 (1985) 5895-5906).

These data are consistent with the hybridization data, described in EP 334462, where no hybridization can be found for P. fragi , P. cepacia (not shown), P. glumae (or P. gladioli), S. hyicus DNA, whereas a proper hybridization signal is obtained from P. aeruginosa DNA. Results are shown in Table 3.

Sequence comparison of the lipase modulator gene

Lipase modulator genes have only been described for Pseudomonas species belonging to RNA homology group II. The lipase modulator gene for P. cepacia (both in EP 331376 and by Jorgensen et al., J. Bacteriol. 173 (1991) 559-567) and the lipase modulator gene of P. glumae (PCT 91/00910) was described. Recently the lipase modulator gene sequence was described (Ihara et al., J. Ferm. Bioeng. 73 (1992) 337-342) of a Pseudomonas species, which might belong to Pseudomonas RNA homology group I, based on sequence homology. Results are shown in Table 4.

Table 3

No.	Strain	1	2	3	4	5	6
1	P.pseudoalcalig.	100	71	70	40	38	41
2	P. aeruginosa	81	100	100	37	41	36
3	P. species	81	100	100	40	38	41
4	P. cepacia	52	41	53	100	33	78
5	P. fragi	56	51	56	46	100	34
6	P. glumae	59	41	59	82	52	100

Table 3 percentage identity of different Pseudomonas lipases. The lower part of the table shows a nucleic acid sequence comparison. The upper part shows an amino acid sequence comparison.

Table 4

No.	Strain	1	2	3	4	5	6
1	P. pseudoalcali.	100	nd	56	33	nd	33
2	P. aeruginosa	nd	100	nd	nd	nd	nd
3	P. species	62	nd	100	32	nd	32
4	P. cepacia	51	nd	53	100	nd	58
5	P. fragi	nd	nd	nd	nd	100	nd
6	P. glumae	51	nd	53	71	nd	100

Table 4 percentage identity between different Pseudomonas lipase modulator genes. The lower part of the table shows a nucleic acid sequence comparison. The upper part shows an amino acid sequence comparison.
nd => for these species no lipase modulator gene was described.

From these data it can be concluded that the lipase modulator genes seem to be less conserved than the lipase genes itself.

It was established that the degree of homology which was found could even be coincidental. An amino acid homology of 25% was found when the lipase sequence of P. pseudoalcaligenes M1 was compared to the lipase modulator sequence of P. pseudoalcaligenes M1, indicating that the observed sequence homology between lipase modulator genes belonging to different RNA homology groups is rather low.

Furthermore Ihara et al. seem to be the first describing the necessity of a modulator gene for Pseudomonas species derived from RNA homology group I in heterologous organisms. For both P. fragi and P. pseudoalcaligenes M1

lipase expression in E. coli didn't seem to depend upon the presence of such a modulating gene.

Based on the very low levels of homology it is rather surprising to be dealing with a gene with a comparable function.

Although the modulator gene, derived from P. pseudoalcaligenes M1, does not seem to be necessary for gene expression in E. coli, it is necessary for levels of lipase gene expression in P. pseudoalcaligenes M1 above 20-fold. This has not been observed before.

SEQUENCE LISTING

(1) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2417 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Pseudomonas pseudoalcaligenes
(B) STRAIN: M1
(C) INDIVIDUAL ISOLATE: CBS 473.85

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 270..1211

(ix) FEATURE:
(A) NAME/KEY: sig_peptide
(B) LOCATION: 270..341

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 342..1208
(C) IDENTIFICATION METHOD: experimental
(D) OTHER INFORMATION: /codon_start= 271
/function= "triglyceride hydrolysis"
/product= "lipase M1"
/evidence= EXPERIMENTAL
/gene= "lip"

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1264..2298
(D) OTHER INFORMATION: /codon_start= 1264
/function= "Lipase modulator"
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AAACGCCCGT TTAGAGCCTT TGTTCTAATC CACCCCGTTC CTGGCACAGA TCCTGCCCCA     180

CCGAGCCTGC TGAAGTACCG GCCCGGGAAG CGCCGGATGG CTGGATGCAA GGATGGATCA     240

GTGCCCAACC CTTCGCTCGA GAGCAAAAC ATG AAT AAC AAG AAA ACC CTG CTC        293
                                Met Asn Asn Lys Lys Thr Leu Leu
                               -24             -20

GCC CTC TGC ATC GGC AGC AGT CTG CTG CTG TCC GGC CCA GCC GAA GCC        341
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  1               5                  10                  15

CTG ACC CAC GGC ATG CTC GGC TTC GAC AGC ATC CTC GGC GTC GAC TAC        437
Leu Thr His Gly Met Leu Gly Phe Asp Ser Ile Leu Gly Val Asp Tyr
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TGG TAC GGC ATC CCG TCC TCG CTG CGC TCC GAC GGC GCC AGC GTC TAC        485
Trp Tyr Gly Ile Pro Ser Ser Leu Arg Ser Asp Gly Ala Ser Val Tyr
        35                  40                  45

ATC ACC GAA GTC AGC CAG CTC AAC ACC TCC GAG CTG CGC GGC GAG GAG        533
Ile Thr Glu Val Ser Gln Leu Asn Thr Ser Glu Leu Arg Gly Glu Glu
    50                  55                  60

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65                  70                  75                  80

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                85                  90                  95

GCC GCC GTA CGC CCG GAC CTG GTG GCC TCG GTG ACC AGC GTC GGC GCC        677
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            100                 105                 110

CCG CAC AAG GGC TCG GAC ACC GCC GAC TTC ATC CGC CAG ATC CCC CCG        725
Pro His Lys Gly Ser Asp Thr Ala Asp Phe Ile Arg Gln Ile Pro Pro
        115                 120                 125

GGC TCG GCC GGT GAG GCG ATA GTC GCC GGC ATC GTC AAC GGC CTG GGC        773
Gly Ser Ala Gly Glu Ala Ile Val Ala Gly Ile Val Asn Gly Leu Gly
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GCC CTG GGC GCC CTC GAA TCG CTC AAC AGT GAG GGC GCC GCC GCC TTC      869
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AAC GCC AAG TAT CCG CAG GGC ATT CCG ACC AGT GCC TGC GGC GAA GGC      917
Asn Ala Lys Tyr Pro Gln Gly Ile Pro Thr Ser Ala Cys Gly Glu Gly
            180                 185                 190

GCC TAC AAG GTC AAT GGC GTC AGC TAC TAC TCC TGG AGC GGC ACC AGC      965
Ala Tyr Lys Val Asn Gly Val Ser Tyr Tyr Ser Trp Ser Gly Thr Ser
        195                 200                 205

CCG CTG ACC AAT GTG CTC GAC GTC AGC GAC CTG CTG CTG GGC GCC AGC      1013
Pro Leu Thr Asn Val Leu Asp Val Ser Asp Leu Leu Leu Gly Ala Ser
    210                 215                 220

TCG CTG ACC TTC GAC GAG CCC AAC GAC GGC CTG GTC GGG CGC TGC AGC      1061
Ser Leu Thr Phe Asp Glu Pro Asn Asp Gly Leu Val Gly Arg Cys Ser
225                 230                 235                 240

TCG CAC CTG GGC AAG GTG ATC CGC GAC GAC TAC CGG ATG AAC CAC CTC      1109
Ser His Leu Gly Lys Val Ile Arg Asp Asp Tyr Arg Met Asn His Leu
                245                 250                 255

GAC GAG GTC AAC CAG ACC TTC GGC CTG ACC AGC CTG TTC GAG ACC GAC      1157
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Pro Val Thr Val Tyr Arg Gln Gln Ala Asn Arg Leu Lys Leu Ala Gly
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Leu
    290

GTG AAC AAA GCC CTG CTT CTG GCC GTA CCC CTG CTG ATC GGG GCC GGC      1311
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  1                 5                  10                  15

ATC GCC GTC ACC CTC GCC CTC AAC CCA CTG ACT CCA GCA CCC AGC CCA      1359
Ile Ala Val Thr Leu Ala Leu Asn Pro Leu Thr Pro Ala Pro Ser Pro
             20                  25                  30

GCG GCG CTA TCG ACT GCG CCT GGC GTA CCG CTG CCG TCG CCA GCG GTG      1407
Ala Ala Leu Ser Thr Ala Pro Gly Val Pro Leu Pro Ser Pro Ala Val
         35                  40                  45
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Gln Arg Thr Leu Asp Asp Ala Pro Ala Ala Pro Pro Leu Ala Ala Glu
     50                  55                  60

ATC GCG CCC CTG CCA CCC TCC TTC GCC GGA ACC CAG GTG GAT GGC CAG      1503
Ile Ala Pro Leu Pro Pro Ser Phe Ala Gly Thr Gln Val Asp Gly Gln
 65                  70                  75                  80

TTC CGC CTC GAT GCG GCA GGC AAC CTG CTG ATC GAA CGG GAT ATC CGG      1551
Phe Arg Leu Asp Ala Ala Gly Asn Leu Leu Ile Glu Arg Asp Ile Arg
                 85                  90                  95

CGC ATC TTC GAC TAC TTC CTC AGC GCC TAT GGC GAG GAC AGC CTC AAG      1599
Arg Ile Phe Asp Tyr Phe Leu Ser Ala Tyr Gly Glu Asp Ser Leu Lys
            100                 105                 110

GCC ACC ATC GAG CGT CTG CAG GCC TAT GTC CGC AGC CAG CTC GAC GAG      1647
Ala Thr Ile Glu Arg Leu Gln Ala Tyr Val Arg Ser Gln Leu Asp Glu
        115                 120                 125

CCG GCC GAA AGC CAG GCC CTG GCG CTG CTG GAG CAG TAC CTG GAG TAC      1695
Pro Ala Glu Ser Gln Ala Leu Ala Leu Leu Glu Gln Tyr Leu Glu Tyr
    130                 135                 140

AAG CGC CAA CTG GTG CAA CTG GAG AAG GAC CTG CCG CAG ATG GCC AGC      1743
Lys Arg Gln Leu Val Gln Leu Glu Lys Asp Leu Pro Gln Met Ala Ser
145                 150                 155                 160

CTG GAT GCC CTG CGT CAG CGC GAG CAG GCG GTG CAG AAC CTG CGT GCC      1791
Leu Asp Ala Leu Arg Gln Arg Glu Gln Ala Val Gln Asn Leu Arg Ala
                165                 170                 175

AGC CTG TTC AGC GTC GAA GCG CAC CAG GCC TTC TTC GCC GAG GAA GAG      1839
Ser Leu Phe Ser Val Glu Ala His Gln Ala Phe Phe Ala Glu Glu Glu
            180                 185                 190

GCC TAC AAC GGC TTC ACC CTG CAG CGC CTG GCG ATC CGT CAC GAC CAG      1887
Ala Tyr Asn Gly Phe Thr Leu Gln Arg Leu Ala Ile Arg His Asp Gln
        195                 200                 205

ACG CTG GAC GAC CAG CAG AAG GCC GAG GCG CTC GAC CGC CTG CGT GCC      1935
Thr Leu Asp Asp Gln Gln Lys Ala Glu Ala Leu Asp Arg Leu Arg Ala
    210                 215                 220

AGC CTG CCG GAA GAG CTA CAG GCA TTG CTG GCC CCG CAG CTG CAG GCC      1983
Ser Leu Pro Glu Glu Leu Gln Ala Leu Leu Ala Pro Gln Leu Gln Ala
225                 230                 235                 240

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CGC TTC GAC GAC AAC GAG CGC CTG CGC CTG GAA GCG GCC GAA CAG CTG      2271
Arg Phe Asp Asp Asn Glu Arg Leu Arg Leu Glu Ala Ala Glu Gln Leu
                325                 330                 335

GCG CAG AGC CGG GAG GAG AAA CCC TGAATGCAAA AAGGCCCGCT TTCGCGGGCC     2325
Ala Gln Ser Arg Glu Glu Lys Pro
            340                 345

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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 313 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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-5 1 5

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10 15 20

Asp Ser Ile Leu Gly Val Asp Tyr Trp Tyr Gly Ile Pro Ser Ser Leu
25 30 35 40

Arg Ser Asp Gly Ala Ser Val Tyr Ile Thr Glu Val Ser Gln Leu Asn
45 50 55

Thr Ser Glu Leu Arg Gly Glu Glu Leu Leu Glu Gln Val Glu Glu Ile
60 65 70

Ala Ala Ile Ser Gly Lys Gly Lys Val Asn Leu Val Gly His Ser His
75 80 85

Gly Gly Pro Thr Val Arg Tyr Val Ala Ala Val Arg Pro Asp Leu Val
90 95 100

Ala Ser Val Thr Ser Val Gly Ala Pro His Lys Gly Ser Asp Thr Ala
105 110 115 120

Asp Phe Ile Arg Gln Ile Pro Pro Gly Ser Ala Gly Glu Ala Ile Val
125 130 135

Ala Gly Ile Val Asn Gly Leu Gly Ala Leu Ile Asn Phe Leu Ser Gly
140 145 150

Ser Ser Ser Thr Ser Pro Gln Asn Ala Leu Gly Ala Leu Glu Ser Leu
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Asn Ser Glu Gly Ala Ala Ala Phe Asn Ala Lys Tyr Pro Gln Gly Ile
170 175 180

Pro Thr Ser Ala Cys Gly Glu Gly Ala Tyr Lys Val Asn Gly Val Ser
185 190 195 200

Tyr Tyr Ser Trp Ser Gly Thr Ser Pro Leu Thr Asn Val Leu Asp Val
205 210 215

Ser Asp Leu Leu Leu Gly Ala Ser Ser Leu Thr Phe Asp Glu Pro Asn
220 225 230

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        235                 240                 245

Asp Asp Tyr Arg Met Asn His Leu Asp Glu Val Asn Gln Thr Phe Gly
    250                 255                 260

Leu Thr Ser Leu Phe Glu Thr Asp Pro Val Thr Val Tyr Arg Gln Gln
265                 270                 275                 280

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INFORMATION FOR SEQ ID NO:3:

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(A) LENGTH: 344 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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Ala Ala Leu Ser Thr Ala Pro Gly Val Pro Leu Pro Ser Pro Ala Val
        35                  40                  45

Gln Arg Thr Leu Asp Asp Ala Pro Ala Ala Pro Pro Leu Ala Ala Glu
    50                  55                  60

Ile Ala Pro Leu Pro Pro Ser Phe Ala Gly Thr Gln Val Asp Gly Gln
65                  70                  75                  80

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                85                  90                  95

Arg Ile Phe Asp Tyr Phe Leu Ser Ala Tyr Gly Glu Asp Ser Leu Lys
            100                 105                 110

Ala Thr Ile Glu Arg Leu Gln Ala Tyr Val Arg Ser Gln Leu Asp Glu
        115                 120                 125

Pro Ala Glu Ser Gln Ala Leu Ala Leu Leu Glu Gln Tyr Leu Glu Tyr
    130                 135                 140

Lys Arg Gln Leu Val Gln Leu Glu Lys Asp Leu Pro Gln Met Ala Ser
145                 150                 155                 160

Leu Asp Ala Leu Arg Gln Arg Glu Gln Ala Val Gln Asn Leu Arg Ala
                165                 170                 175
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Ser Leu Pro Glu Glu Leu Gln Ala Leu Leu Ala Pro Gln Leu Gln Ala
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Glu Leu Arg Gln Gln Thr Ala Ala Leu Gln Ala Gln Gly Ala Ser Ala
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260 265 270

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275 280 285

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290 295 300

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305 310 315 320

Arg Phe Asp Asp Asn Glu Arg Leu Arg Leu Glu Ala Ala Glu Gln Leu
325 330 335

Ala Gln Ser Arg Glu Glu Lys Pro
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Claims

1. A method for obtaining lipases comprising:
- cloning of a lipase gene and a lipase modulator gene obtained from a class I Pseudomonas species in a homologous Pseudomonas species,
- culturing of the recombinant strain under conditions wherein the lipase gene is expressed,
- isolating the lipase from the culture.

2. The method according to claim 1, wherein the class I Pseudomonas species is selected from the group consisting of: Pseudomonas alcaligenes, Pseudomonas pseudoalcaligenes, Pseudomonas stutzeri, Pseudomonas aeruginosa, and Pseudomonas mendocina, or mutants thereof.

3. The method according to claim 1, wherein the class I Pseudomonas species is Pseudomonas pseudoalcaligenes or mutants thereof.

4. The method of any one of the previous claims wherein the class I Pseudomonas species host strain is lipase and/or lipase modulator deficient.

5. A method for increasing the lipase production of class I Pseudomonas strains comprising the cloning and expression of both a class I lipase and lipase modulator gene in the host cell.

6. A class I Pseudomonas strain transformed with a lipase and a lipase modulator gene obtained from the homologous strain.

7. A Pseudomonas strain transformed with a vector containing a lipase gene wherein said strain is characterized in that the amount of lipase produced is at least 15-fold higher than in the untransformed strain.

8. A Pseudomonas strain according to claim 7 wherein the strain is additionally transformed with a lipase modulator gene.

9. A Pseudomonas strain according to claim 7 or 8 selected from the class I Pseudomonas and wherein the strain is transformed with a homologous lipase and/or lipase modulator gene.

10. A derivative of pJRD215 which is segregationally stable in Pseudomonas.

11. A method for obtaining a segregationally stable derivative of pJRD215 comprising:
- repeated dilution of the transformed Pseudomonas strain in medium without antibiotics
- followed by incubation periods in the presence of antibiotics.

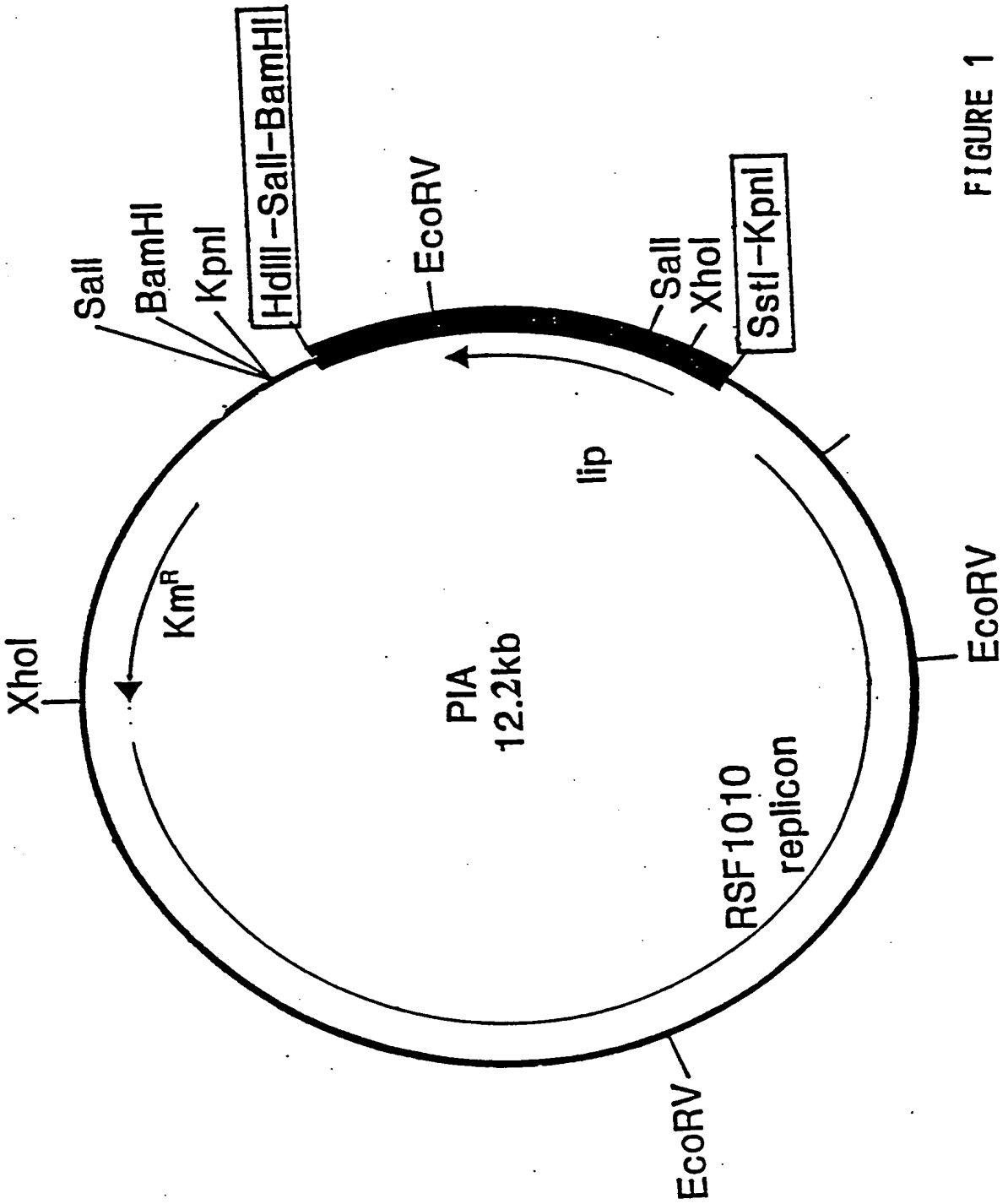
SalI
BamHI
KpnI
HdIII–SalI–BamHI
EcoRV
SalI
XhoI
SstI–KpnI
lip
EcoRV
EcoRV
RSF1010
replicon
PIA
12.2kb
KmR
XhoI

FIGURE 1

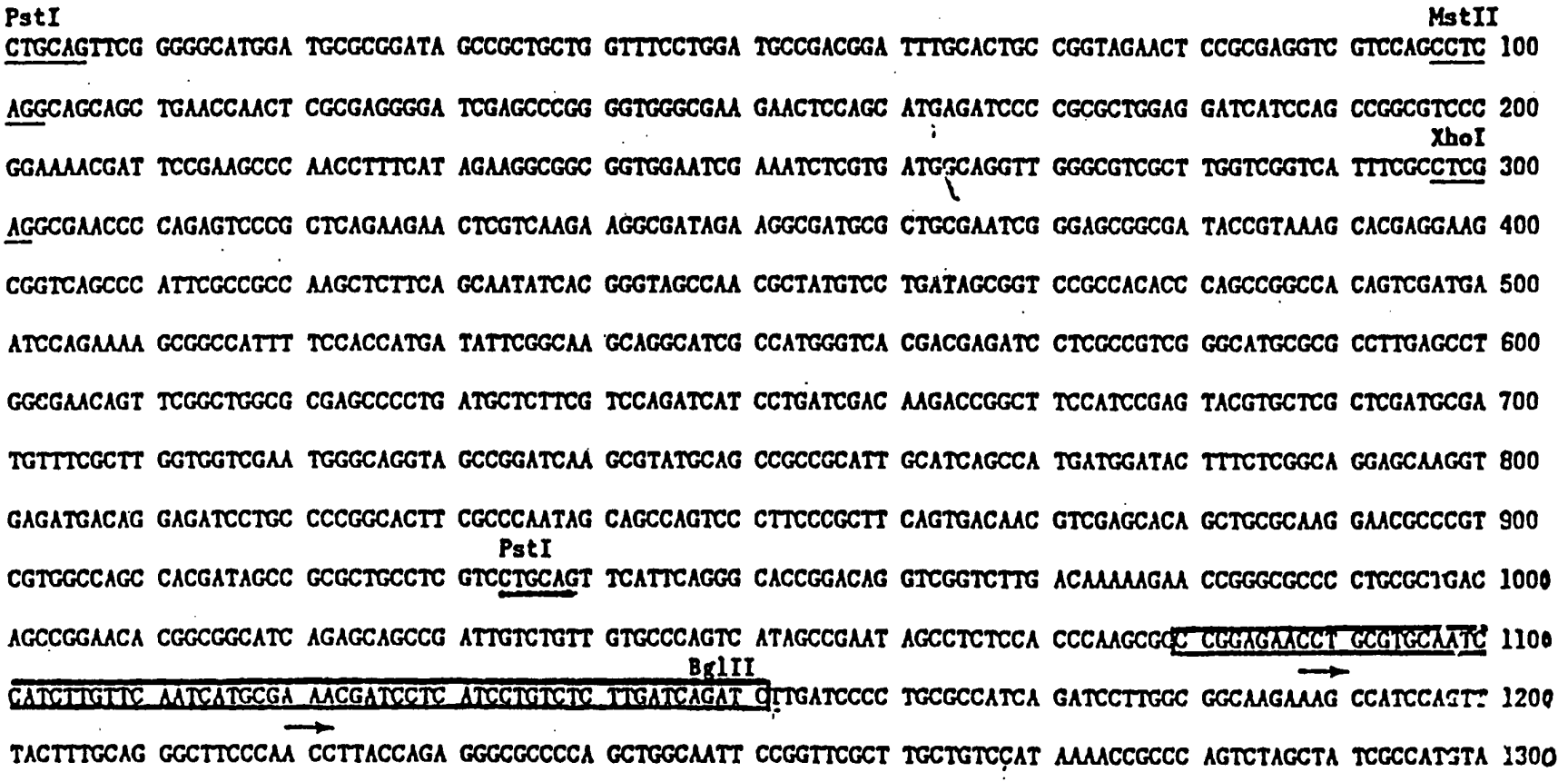

FIGURE 2 A

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                                                                    NheI       PstI
GACTGGCTTT CTACGTGTTC CGCTTCCTTT AGCAGCCCTT GCGCCCTGAG TGCTTGCGGC AGCGTGAAGC TAGCTTGGCT GCAGGGGGGG GGGGGGACT 1500

TCCATTGTTC ATTCCACGGA CAAAAACAGA GAAAGGAAAC GACAGAGGCC AAAAAGCCTC GCTTTCAGCA CCTGTCGTTT CCTTTCTTTT CAGAGGGTAT 1600
DraI
TTTAAATAAA AACATTAAGT TATGACGAAG AAGAACGGAA ACGCCTTAAA CCGGAAAATT TTCATAAATA GCGAAAACCC GCGAGGTCGC CGCCCCGTAA 1700

CCTGTCGGAT CACCGGAAAG GACCCGTAAA GTGATAATGA TTATCATCTA CATATCACAA CGTGCGTGGA GGCCATCAAA CCACGTCAAA TAATCAATTA 1800

TGACGCAGGT ATCGTATTAA TTGATCTGCA TCAACTTAAC GTAAAAACAA CTTCAGACAA TACAAATCAG CGACACTGAA TACGGGGCAA CCTCATGTCC 1900
          PstI SalI   BamHI     KpnI
CCCCCCCCCC CTGCAGGTCG ACGGATCCCC GGGTACCATA AGTAGAAGCA GCAACCCAAG TAGCTTTACC AGCATCCGGT TCAACCAGCA TAGTAAGAAT 2000
                      AsuII                  PstI
CTTACTGGAC ATCGGCAGTT CTTCGAACAG TGCGCCAACT ACCAGCTCTT TCTGCAGTTC ATTCAGGGCA CCGGAGAACC TGCGTGCAAT CCATCTTGTT 2100
                                    MluIHpaI     ApaI       NsiINdeI    →  StuI      XbaI
CAATCATGCG AAACGATCCT CATCCTGTCT CTTGATCCAT GGATTACGCG TTAACCCGGG CCCGCGGATG CATATGATCT TAAGGCCTAG GTCTAGAGTC 2200
          →          SnaBI                                                          SpeI
TTTGTTTTGA CGCCATTAGC GTACGTAACA ATCCTCGTTA AAGGACAAGG ACCTGAGCGG AAGTGTATCG TACAGTAGAC GGAGTATACT AGTATAGTCT 2300
                                                  EcoRI                      ClaI   HindIII
ATAGTCCGTG GAATTATTAT ATTTATCTCC GACGATATTC TCATCAGTGA AATCCAGGGG AATTCTCATG TTTGACAGCT TATCATCGAT AAGCTTTAAT 2400

GCGGTAGTTT ATCACAGTTA AATTGCTAAC GCAGTCAGGC ACCGTGTATG AAATCTAACA ATGCGCTCAT CGTCATCCTC GGCACCGTCA CCCTGGATGC 2500

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FIGURE 2 B

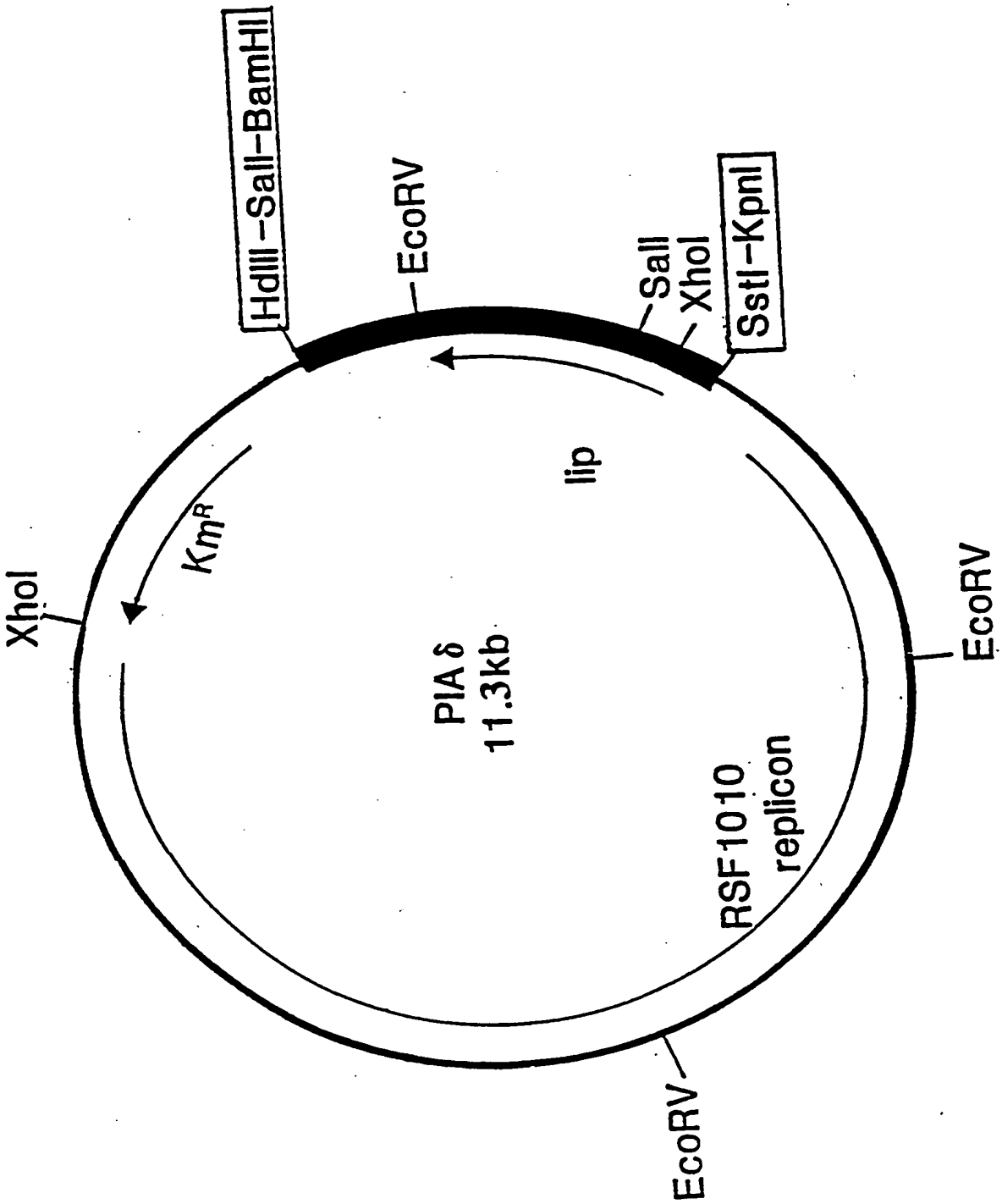
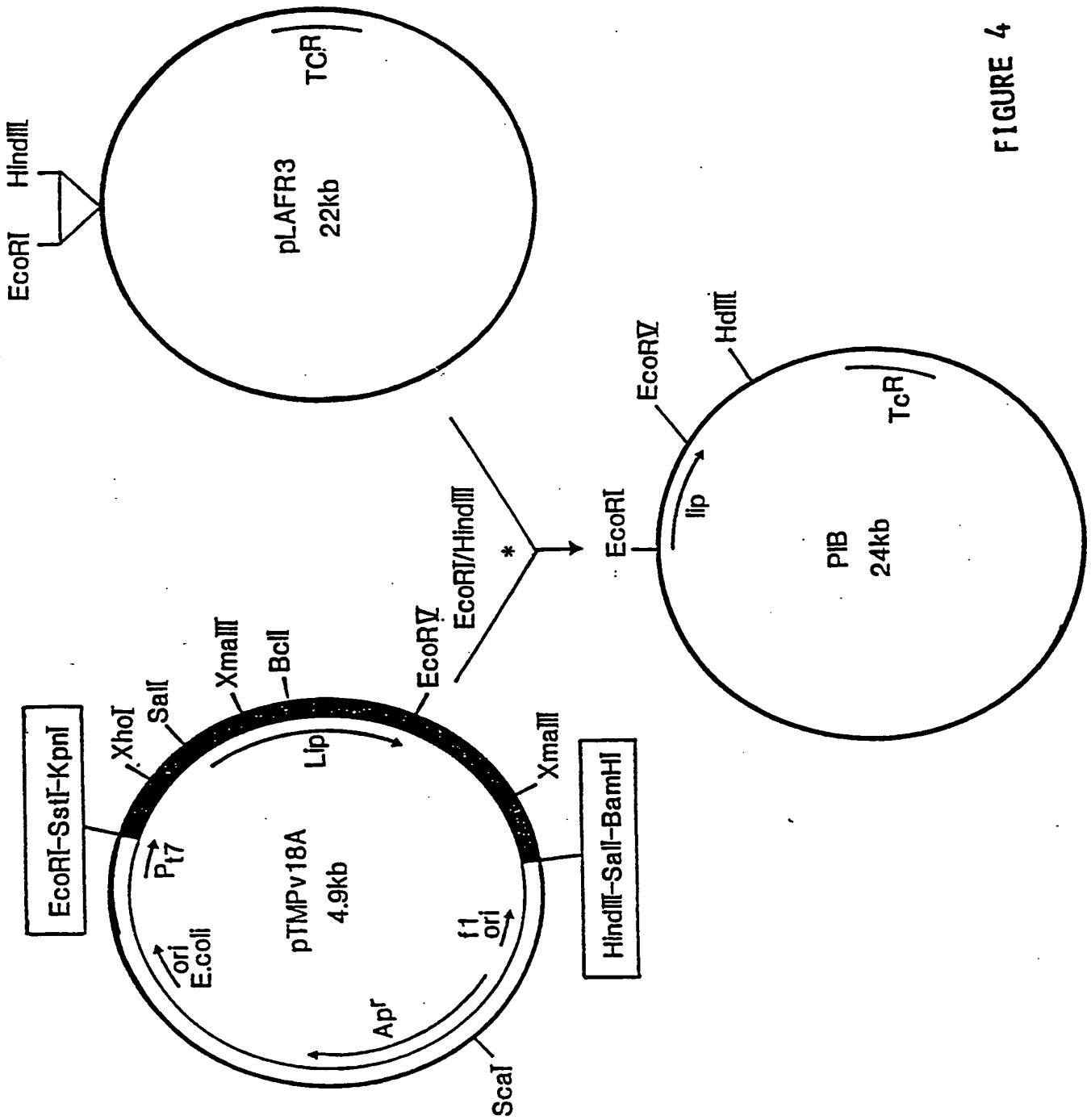


FIGURE 3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HindIII
pLAFR3
22kb
TCR
EcoRI/HindIII
*
EcoRI
EcoRV
HdIII
lip
PIB
24kb
TcR
EcoRI-SstI-KpnI
XhoI
SalI
XmaIII
BclI
EcoRV
Lip
XmaIII
HindIII-SalI-BamHI
Pt7
pTMPv18A
4.9kb
ori
E.coli
f1
ori
Apr
ScaI

FIGURE 4

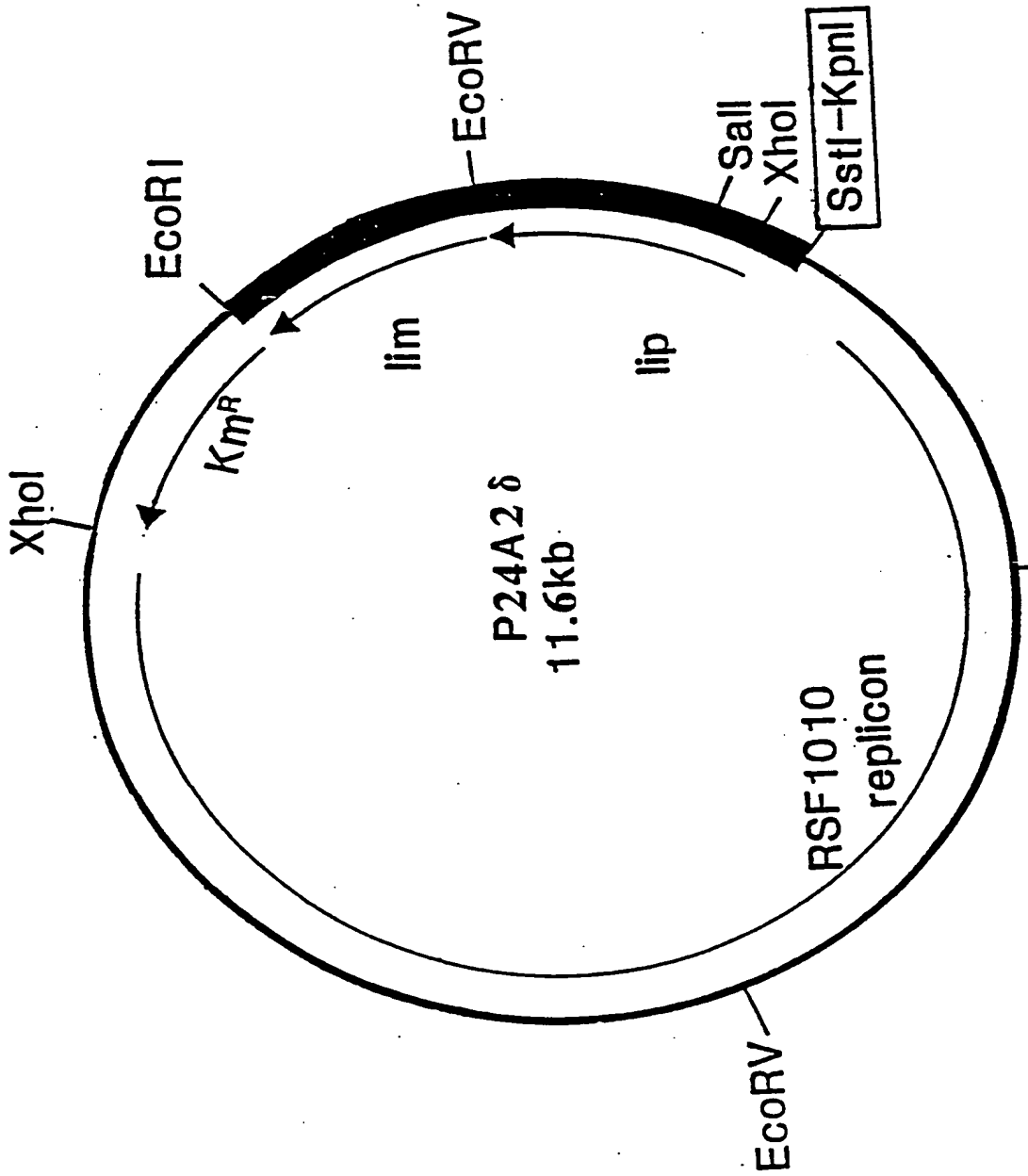

FIGURE 5

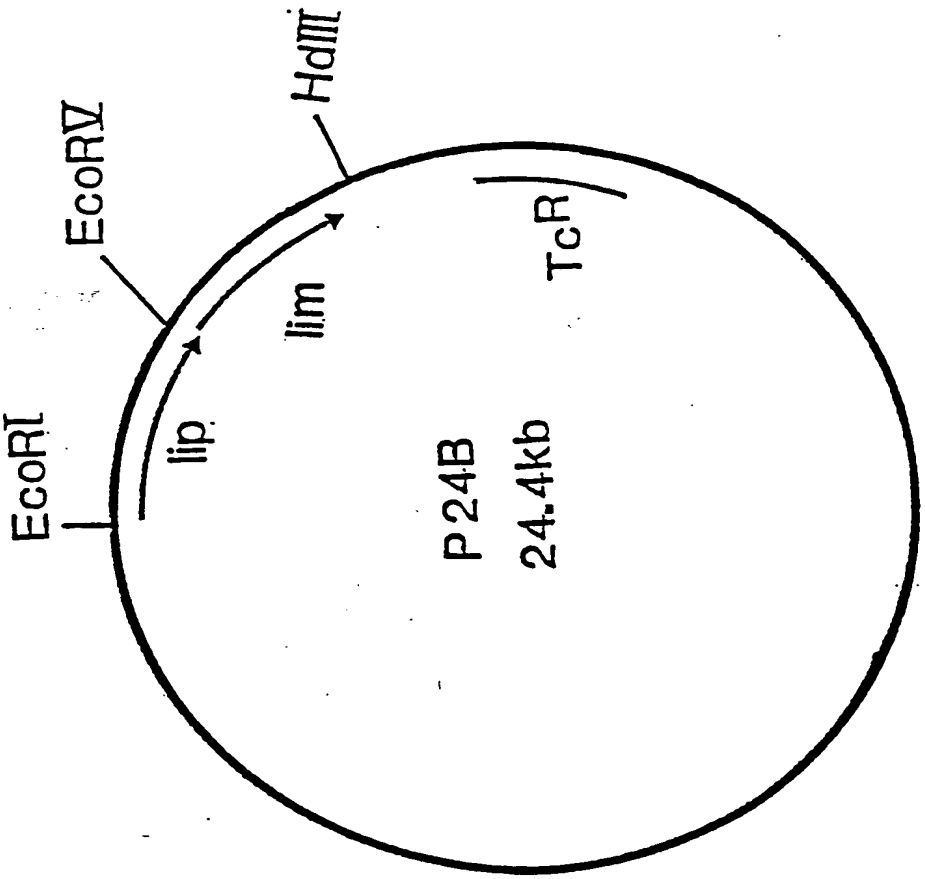

FIGURE 6

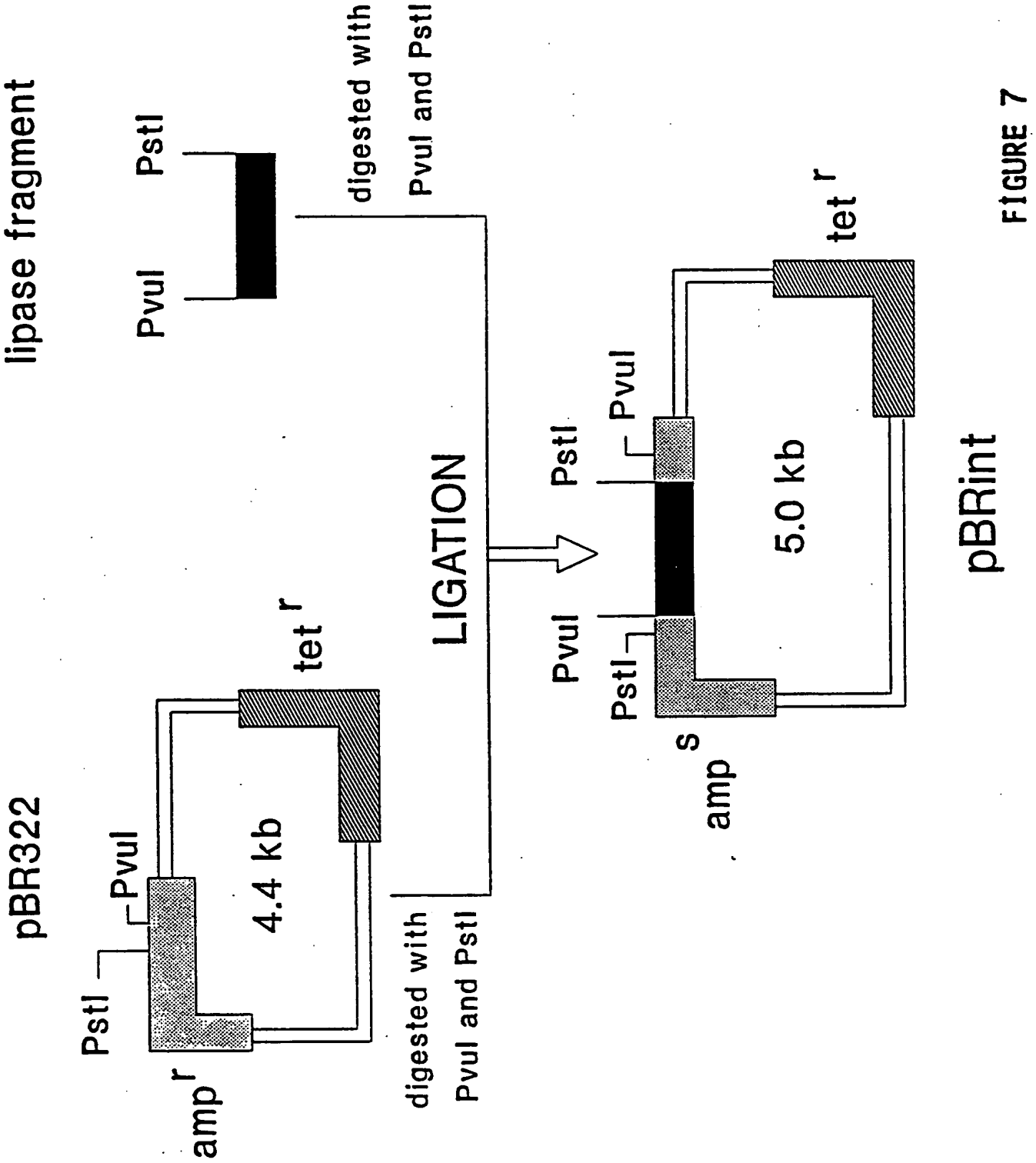

FIGURE 7

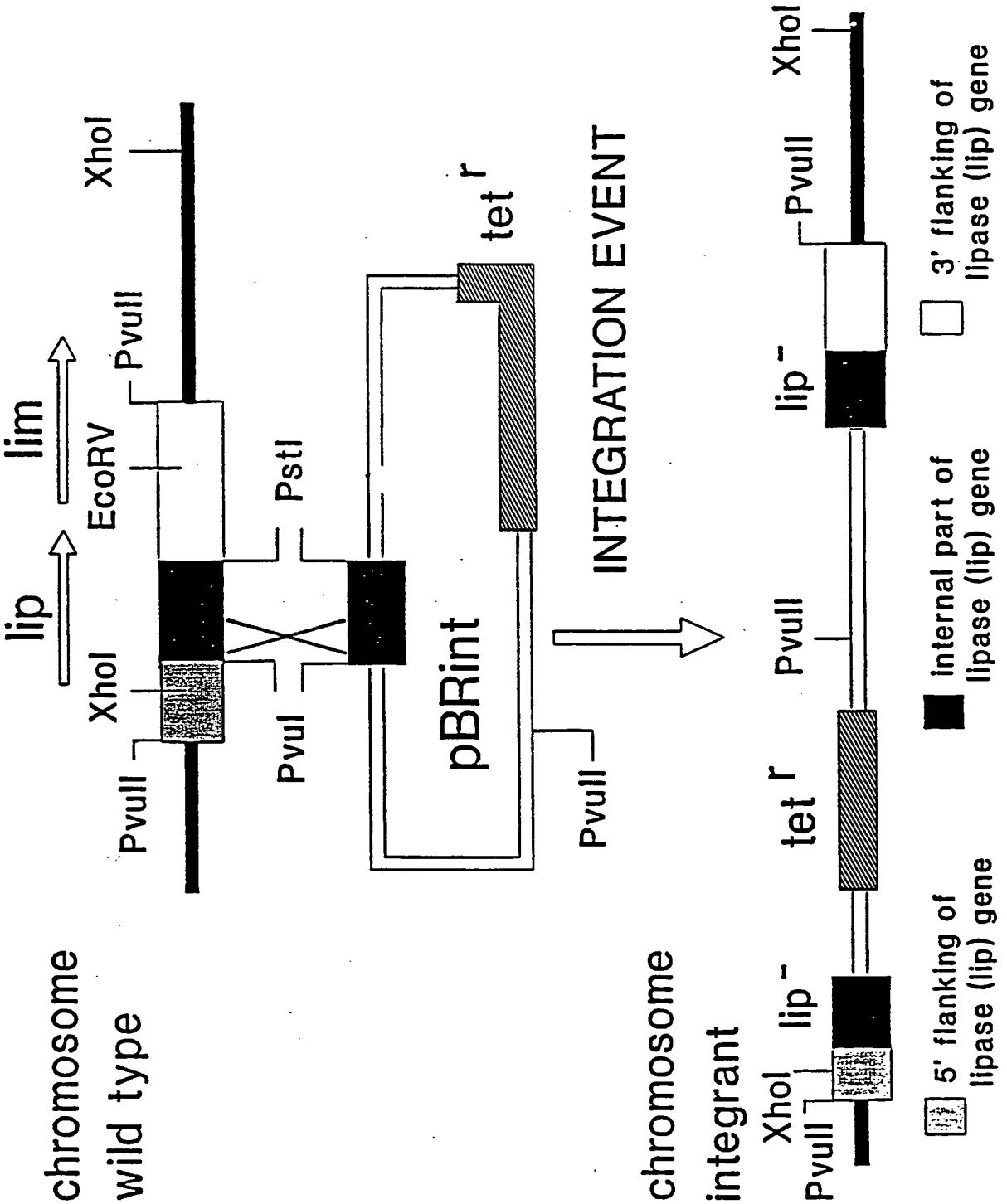
chromosome
wild type
lip
lim
XhoI
PvuII
EcoRV
PstI
XhoI
PvuII
PvuI
pBRint
tet r
PvuII
INTEGRATION EVENT
chromosome
integrant
XhoI
lip -
PvuII
tet r
lip -
PvuII
XhoI
5' flanking of lipase (lip) gene
internal part of lipase (lip) gene
3' flanking of lipase (lip) gene

FIGURE 8

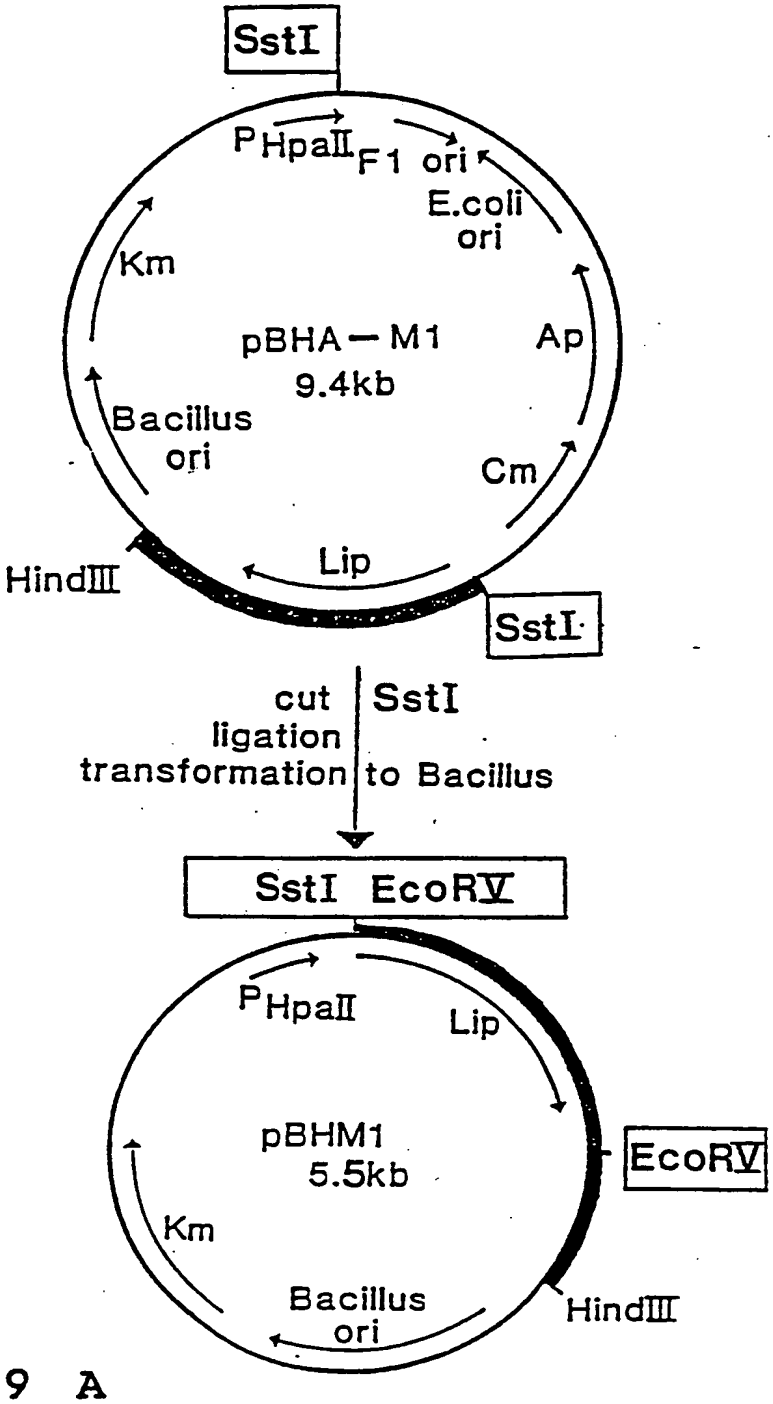

FIGURE 9 A

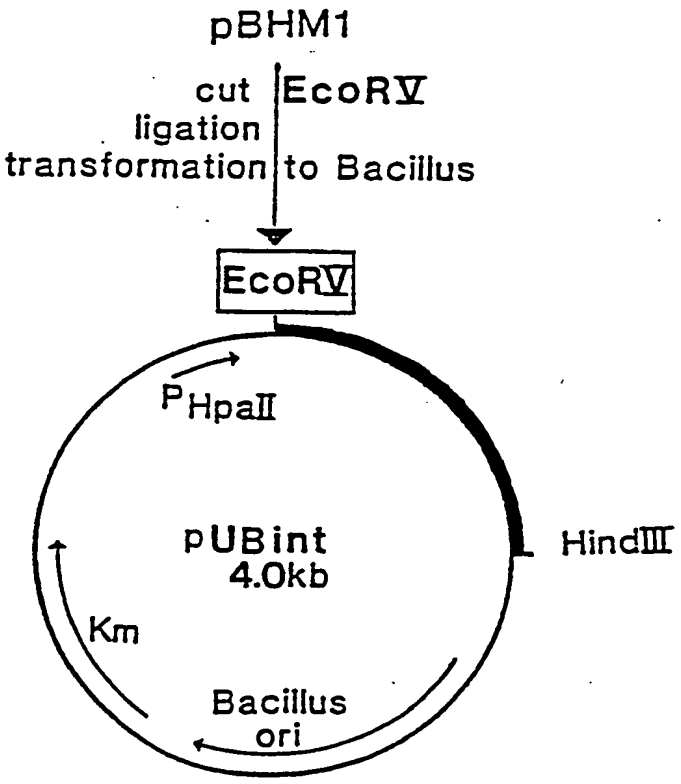
pBHM1
cut EcoRV
ligation
transformation to Bacillus
EcoRV
PHpaII
pUBint
4.0kb
HindIII
Km
Bacillus
ori

FIGURE 9 B

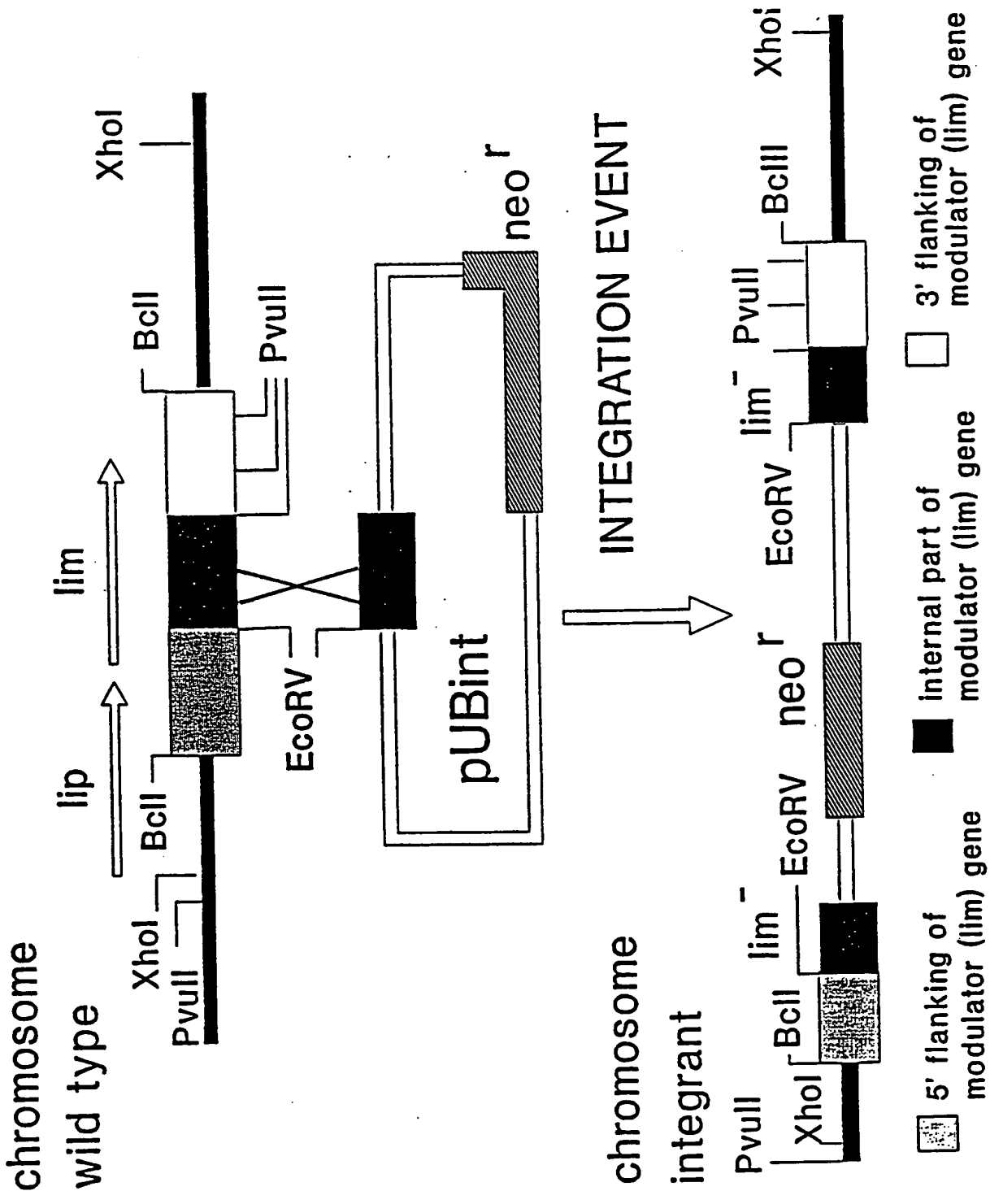

FIGURE 10